Study of DNA Methylation by Tobacco-Specific *N*-Nitrosamines

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An enzyme-linked immunosorbent assay (BA-ELISA) involving use of biotin-labeled anti-rabbit IgG and avidin-labeled horseradish peroxidase was developed for the measurement of O^6-methyl-2'-deoxyguanosine (O^6-MedGuo). Up to 5 µg of methylated DNA was enzymatically hydrolyzed, and the extent of inhibition of binding of immobilized O^6-MedGuo-bovine serum albumin to rabbit anti-O^6-MedGuo was measured. Fifty percent inhibition of antigen–antibody binding was achieved with 2.5 pmole of of O^6-MedGuo. Separation of O^6-MedGuo from unmodified nucleosides by high-performance liquid chromatography (HPLC-BA-ELISA) allowed detection of 700 fmole O^6-MedGuo in 1 mg of DNA.

Among the tobacco-related carcinogens, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the most potent. In F344 rats it induces nasal cavity, lung and liver tumors. Four hours after a single IV injection of NNK to F344 rats (87 mg/kg body weight), O^6-MedGuo was present in target organs (µmole O^6-MedGuo/mole dGuo) (nasal mucosa, 219; lung, 13.2; and liver, 34.5) but was not detectable in nontarget organs. F344 rats receiving daily IP injections of NNK (40 mg/kg body weight) for 14 days were sacrificed 24 hr after the last injection. The levels of (O^6-Medguo/dGuo) were 7.9 and 11.4 µmole/mole in the nasal mucosa and lung, respectively. In the liver no O^6-MedGuo was detected, but 1050 µmole of 7-MeGua/mole Gua was measured by HPLC-fluorimetry. No DNA methylation was observed in the nasal mucosa or liver of F344 rats treated with the nicotine-derived carcinogen *N*'-nitrosonornicotine. Reduction of the carbonyl of NNK is a major metabolic pathway, giving rise to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butan-1-ol (NNAl). Nasal mucosae were cultured *in vitro* with NNK or NNAl. After 1 hr, methylation at the O^6-dGuo and 7-dGuo sites were observed with NNK but not with NNAl. Methylation by NNAl after 24 hr was associated with the conversion of NNAl to NNK. These results suggest that NNAl is not associated with the activation of NNK to DNA methylating species.

Introduction

The high incidence of lung cancer among smokers is well documented (1). Studies carried out during the last 10 years have shown that *N*-nitrosamines present in tobacco smoke are relatively potent carcinogens in animals. The same studies have suggested that *N*-nitrosamines are important etiological factors in tobacco smoking-related cancer (2). Among the 13 *N*-nitrosamines detected in cigarette smoke, the tobacco-specific *N*-nitrosamines are among the most abundant (3). Prospective and retrospective studies have demonstrated a link between snuff exposure and development of oral cancer (4,5). At present, tobacco-specific *N*-nitrosamines are the only carcinogens to have been isolated in significant amounts from snuff (6). The levels of ab-

sorption of tobacco components by respiratory tract tissues vary among smokers and intrinsic exposure to *N*-nitrosamines during tobacco smoking is difficult to assess. The tobacco-specific *N*-nitrosamines: *N*'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are derived from nicotine and are metabolized rapidly *in vivo* to metabolites identical to those of nicotine (2). Consequently, an assessment of exposure to nicotine-derived *N*-nitrosamines by quantification of their metabolites is not feasible.

However, alkylating species are generated only from NNN and NNK, and levels of their persistent adducts in DNA and proteins could be used as an index of human exposure. Alternatively, assessment of repair of promutagenic DNA damage would measure the ability of an individual to respond to carcinogen insults. The objective of the present study was to develop a safe, inexpensive and sensitive method to measure the levels of O^6-methyl-2'-deoxyguanosine (O^6-MedGuo) in DNA damaged by activated NNK.

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Materials and Methods

Chemicals

Synthesis of NNK has been described, and its purity was greater than 99% by high performance liquid chromatography (HPLC) (7). Sodium borohydride reduction of NNK gave 4-(methylnitrosamino)-1-(3-pyridyl)-butan-1-ol (NNAl) which was free of any traces of NNK by reverse phase HPLC.

Culture of Rat Nasal Mucosa. Removal of the nasal septum and its covering mucosa from the rat nasal cavity and the culture technique of the septum have previously been described (8). Each septum was cultured in a 35-mm culture dish (Falcon, Bethesda, MD) with 2 mL of medium containing either 100 µg of NNK or 101 µg of NNAl/mL. After 1, 3, or 24 hr of culture, the septa were harvested, and DNA was extracted from three combined explants. The medium was harvested and stored frozen. After filtration of the media, levels of NNK and NNAl were measured by HPLC on an octodecylsilane-bonded phase column (4.6 mm × 12.5 cm) as described elsewhere (9).

Rat Treatment with NNK and NNN. Five F344 rats each weighing approximately 280 g at the start of the experiment were fed an NIH-07 diet (Ziegler Brothers, Inc., Gardners, PA). They were injected IV with a solution of NNK in 0.9% $NaCl/H_2O$ (87 mg/kg body weight) and decapitated 4 hr later. Livers, lungs, kidneys, esophagi, spleens, and hearts were excised and immediately frozen over dry ice. The nasal cavity was opened as described previously (8), and the mucosa covering the nasal septa and ethmoturbinates of the five rats was scraped off with a scalpel, pooled, and the DNA extracted as described previously (10).

Two F344 rats each weighing about 248 g received each daily IP injections of 10 mg of NNN dissolved in 0.9% $NaCl/H_2O$ (20 mg/mL). Each rat received 14 injections. Two other rats each received 10 mg of NNK dissolved in 0.9% $NaCl/H_2O$ (20 mg/mL). The four rats were exsanguinated by heart puncture 24 hr after the last injection and the tissues mentioned above were excised and stored frozen until DNA extraction.

DNA samples were dissolved in 50 mM pH 7.2 Tris HCl and DNA concentration was determined by fluorimetry (11). DNA was hydrolyzed according to the method of Müller and Rajewsky (12).

Antibody Production and Purification

O^6-Methylguanosine (O^6-MeGuo) was conjugated to bovine serum albumin (BSA) (Sigma, St. Louis, MO) or keyhole limpet hemocyanin (KLH) (Calbiochem, La-Jolla, CA) according to the method of Erlanger and Beiser (13). New Zealand White rabbits were immunized with an emulsion of the conjugate in complete Freuds adjuvant (Calbiochem). The inoculum was injected intradermally at approximately 20 sites on the back of each rabbit. Four booster injections were given at 8-week intervals. The 2-week bleeding after the last booster injection was used in the present study. The anti-O^6-MedGuo antibodies were purified by affinity chromatography as previously described for the anti-O^6-ethyl-2'-deoxyguanosine (12).

BA-ELISA. The biotin-avidin enzyme-linked immunosorbent assay (BA-ELISA) was performed entirely at room temperature in 96-well polystyrene microtiter plates (Costar, Cambridge, MA). Each experimental value was determined in triplicate. The amount of O^6-MedGuo in a sample was determined by the use of two standard curves. The first set of standards was used to measure the extent of inhibition of binding of the primary antibody. The primary antibody was diluted 1:1000. Further dilution of 4:5, 3:5 and 2:5 (corresponding to 20, 40 and 60% inhibition) served to construct the standard curve. The second curve consisted of five 6-fold dilutions of O^6-MedGuo ranging between 0.1 and 25 pmole per well and was used to equate a given amount of O^6-MedGuo with a given degree of inhibition.

All wells were coated with 100 µL of O^6-MeGuo-BSA at a concentration of 100 ng/mL in 50 mM Tris-HCl buffer, pH 7.2, for 1 hr. After the removal of unbound O^6-MeGuo–BSA, unoccupied protein binding sites were saturated by the addition of 100 µL of a 1% (w/v) solution of BSA in phosphate-buffered saline (PBS) for 30 min. Wells were then emptied and washed. The standard washing procedure was to rinse twice with PBS–Tween (Sigma) (0.5 mL Tween 20/L PBS) and four times with distilled water.

To set up the standard curves and experimental points on the plate the following procedure was followed. All reagents were diluted in PBS containing 0.1% (w/v) BSA. O^6-MedGuo standards or experimental samples as 50 µL aliquots were added to the appropriate wells. Wells used for the primary antibody standards received 50 µL of diluent. Then wells containing O^6-MedGuo standards or experimental samples received 50 µL of primary antibody (1:1000 dilution) and wells for the primary antibody standards received 50 µL of the appropriate antibody dilution. After incubation for 1 hr the wells were emptied and washed with PBS as above.

Bound antibody was detected with biotin-labeled anti-rabbit IgG and avidin-labeled horseradish peroxidase (Vector Labs, Burlingame, CA). Then 100 µL of biotin-labeled antibody was incubated in the wells for 1 hr, then removed and the wells washed as before; 100 µL of avidin-horseradish peroxidase was then incubated for 30 min and washed as before. Enzyme activity was measured with the substrate 1,2-diaminobenzene (*o*-phenylenediamine, Sigma) at 500 µg/mL in 100 mM pH 6.2, phosphate buffer with 0.006% H_2O_2 (v/v) or with 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt (Sigma) at 200 µg/mL in 0.01 M phosphate buffer, pH 6.0, with 0.001% H_2O_2 (v/v). Freshly prepared substrate solution, 100 µL, was added to each well and incubated until the absorbance was approximately 0.5. The reaction was stopped with 100 µL of 2 N HCl. The absorbance was read at 490 nm for 1,2-

diaminobenzene and at 405 nm for 2,2'-azino-di-(3-ethyl benzthiazoline sulfonic acid) diammonium salt.

HPLC-BA-ELISA. The HPLC apparatus has been described previously (14). Separation of deoxyribonucleosides obtained by DNA hydrolysis was performed with a μ-Bondapak-C_{18} column (4.9 mm × 30 cm) (Waters Associated, Milford, MA) and with a gradient of 0 to 35% aqueous methanol in 50 min. The flow rate was 1 mL/min, and 1-mL fractions were collected. The fractions were then lyophilyzed and reconstituted with 0.5 mL of PBS. Individual fractions were then analyzed by BA-ELISA as described above. The recovery of O^6-MedGuo from HPLC vary between 70 and 100%. HPLC-fluorimetry was carried out as described by Herron and Shank (15).

Results

Figure 1 demonstrates the specificity and sensitivity of the antibody used in the BA-ELISA for O^6-MedGuo. Fifty percent inhibition of antibody binding to immobilized O^6-MedGuo-BSA was achieved with 2.5 pmole of O^6-MedGuo. If a probability grid was used to plot the inhibition curves, inhibition was linear between 15 and 85%. Fifteen percent inhibition is equivalent to 100 fmole of O^6-MedGuo per well. Since, of the compounds tested, those containing a methyl group attached to the O^6 position of guanine gave the highest levels of inhibition, the specificity of the antibody was clearly directed towards this group. N^6-Methyl-2'-deoxyadenosine (N^6-MedAdo) was at least 1000-fold less efficient than O^6-MedGuo in inhibiting antigen-antibody binding. This indicates that the antibody may recognize the amino group at the 2 position of the O^6-MedGuo. The poor inhibition of binding of 7-methyl-2'-deoxyguanosine (7-MedGuo) versus O^6-MedGuo and of 3-methylguanine versus O^6-MeGua emphasizes the importance of the methyl group being attached to the O^6 position of the guanine moiety. All five nucleosides naturally occurring in DNA were found to inhibit binding only at concentrations 100,000-fold higher than those required for O^6-MedGuo binding.

The maximum amount of hydrolyzed DNA that could be used per well was 5 μg. This maximum limit sets the lower limit of methylation which can be detected for 20 pmole O^6-MedGuo/mg DNA. At 25 pg of DNA per cell this level of methylation corresponds to 316,000 O^6-MedGuo residues per diploid genome (16). To circumvent this limitation, reverse-phase HPLC was used to separate O^6-MedGuo from unmodified and other methylated nucleosides. As shown in Figure 2 this technique was used to measure the level of O^6-MedGuo in DNA isolated from the nasal mucosa of NNK treated rats. Only those fractions having an elution volume equal to that of O^6-MedGuo gave substantial inhibition (64%) of antibody binding in the BA-ELISA. By coupling HPLC with the BA-ELISA the amount of DNA analyzed can be increased from 5 μg to 1 mg or more. Following this procedure a 1 mg sample containing as little as 700 fmole, which corresponds to 10,500 residues of O^6-MedGuo per diploid genome, can be analyzed in duplicate (six wells).
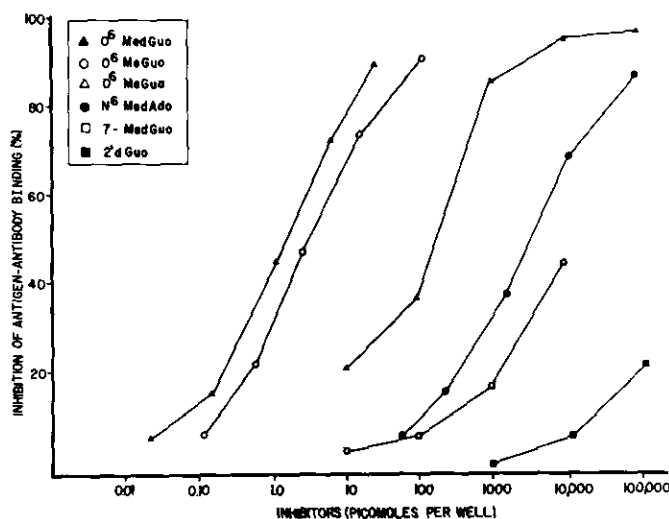

FIGURE 1. Inhibition of anti-O^6-MedGuo antibody binding to immobilized O^6-MeGuo-BSA by various nucleobases and nucleosides. At a concentration of 100,000 pmole per well, 2'-deoxyadenosine gave 39% inhibition, dGuo, adenine, and thymidine gave less than 20% inhibition and 2'-deoxycytosine, 5-methyl-2'-deoxycytosine, 3-methylguanine, and 7-methyladenine gave less than 10% inhibition.
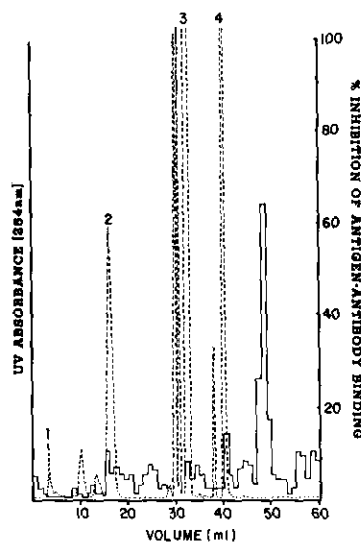
FIGURE 2. HPLC separation of deoxyribonucleosides obtained by enzyme hydrolysis of 367 μg of DNA isolated from the nasal mucosa of F344 rats sacrificed 4 hr after IV injection of NNK (87 mg/kg body weight). Numbered peaks were identified as follows: (1) hydrolyzing enzymes; (2) 2'-deoxycytidine; (3) dGuo and thymidine; (4) 2'-deoxyadenosine. The retention volume of O^6-MedGuo under these conditions is 49 mL. The peak at this retention volume corresponds to 5.2 pmole of O^6-MedGuo: (--)UV (254 nm); (———) inhibition of antigen–antibody binding.

The accuracy of BA-ELISA and HPLC-BA-ELISA methods was compared to that of radiochromatographic and HPLC-fluorimetric methods. Calf thymus DNA was methylated with [$^{14}CH_3$]N-methyl-N-nitrosourea and analyzed by the four methods. The methods agree well with each other, giving correlation coefficients by linear regression analysis greater than 0.99.

FIGURE 3. Metabolic activation of NNK to methylating intermediates by target tissues of F344 rats. Structures in brackets are hypothetical intermediates.

The metabolic pathways of NNK are illustrated in Figure 3. Carbonyl reduction of NNK gives NNAl. As with most N-nitrosamines, hydroxylation of the carbon α to the N-nitroso group of NNK and NNAl could be considered a crucial and rate-limiting step in their bioactivation to alkylating species. For instance, α-methylene hydroxylation of NNK and NNAl would lead to the generation of methyldiazohydroxide (7). The keto acid and hydroxy acid (compounds 10 and 11 of Fig. 3) are two major urinary metabolites of F344 rats treated with NNK (17).

The methylating capacities of NNK and NNAl were compared by culturing rat nasal mucosa with these two N-nitrosamines. As shown in Table 1, NNK was rapidly reduced to NNAl in this system. Only 14% of the initial amount of NNK remained at the end of the 24-hr culture period. In contrast, NNAl was not extensively metabolized, and 77% of the initial amount remained unchanged after 24 hr.

After 24 hr of culture, the total level of NNK + NNAl was 107 nmole/mL and 373 nmole/mL when NNK and NNAl were used as starting N-nitrosamines, respectively. The extent of DNA methylation was higher when NNK rather than NNAl was added to the culture medium (Table 2). With NNAl, DNA methylation after 24 hr was associated with the oxidation of a small fraction of NNAl to NNK.

Table 2. Levels of methylation of rat nasal mucosa cultured *in vitro* with NNK or NNAl.

	Starting N-nitrosamine[a]			
	NNK		NNAl	
Time of culture, hr	O^6-MeGua, μmole/mole Gua	7-MeGua, μmole/mole Gua	O^6-MeGua, μmole/mole Gua	7-MeGua, μmole/mole Gua
1	39	600	ND[b]	ND
3	65	1060	ND	—[c]
24	138	—	52.1	745

[a]Mean of two samples of DNA extracted from three nasal septa each. The initial concentrations of NNK or NNAl were 482 nmole/mL.
[b]Not detected. Limit of detection is 30 μmole/mole dGuo.
[c]Not determined.

F344 rats were either treated with a single dose of NNK and sacrificed 4 hr later or were administered 14 daily doses of NNK and sacrificed 24 hr later. As shown in Table 3, O^6-MedGuo was found only in nasal mucosa, lung and liver.

Table 1. NNK-NNAl interconversions during culture of rat nasal mucosa with either NNK or NNAl.

	Starting N-nitrosamine[a]			
	NNK		NNAl	
Time of culture, hr	NNK, nmole/mL	NNAl, nmole/mL	NNK, nmole/mL	NNAl nmole/mL
1	440 ± 7	35 ± 5	ND[b]	480 ± 11
3	323 ± 8	80 ± 5	2.6 ± 0.1	398 ± 10
24	68 ± 9	39 ± 1	4.2 ± 0.3	369 ± 11

[a]The initial concentration of NNK or NNAl was 482 nmole/mL. Values are mean ± SE obtained from four cultured media.
[b]ND = not detected. Limit of detection is 1.2 nmole/mL.

Table 3. Susceptibility of F344 rat organs to develop tumors and levels of O⁶-MedGuo after treatment with NNK.

Organ	Susceptibility[a]	O^6-MedGuo/dGuo, μmole/mole[b]	
		Single dose (87 mg/kg)[c]	Multiple dose (40 mg/kg)[d]
Nasal mucosa	+ +	219 ± 9.9	7.9 ± 1.4
Lung	+ + +	13 ± 1.6	11.4 ± 3.8
Liver	+	34 ± 2.6	ND[e]
Kidney	–	ND[f]	ND
Esophagus	–	ND	ND
Spleen	–	ND	ND
Heart	–	ND	ND

[a] F344 rats were injected SC with 60 subdoses of NNK during 20 weeks. Total dose was 1 mmole/kg body weight (19).

[b] Values are mean ± SE ($n = 3$) as determined by HPLC-BA-ELISA.

[c] Five F344 rats were injected IV with a solution of NNK (87 mg/kg body weight) and were sacrificed 4 hr later.

[d] Two F344 rats received each daily IP injections of NNK (40.3 mg/kg body weight) for 14 days. The rats were sacrificed 24 hr after the last injection.

[e] Level of 7-MeGua/mole Gua as measured by HPLC-fluorimetry was 1050 μmole.

[f] Not detected. Limit of detection was 3 μmole O⁶-MedGuo/mole dGuo.

Discussion

The BA-ELISA described in this report is a sensitive, rapid, and simple method for the detection of O⁶-MedGuo in DNA modified by methylating agents. The use of biotin–avidin reagents reduces nonspecific binding of the second antibody and results in lower background readings. The BA-ELISA can easily be performed in one day and does not require the use of radiolabeled tracers. The sensitivity of BA-ELISA was increased considerably by chromatographic separation of methylated and naturally occurring nucleosides obtained by enzyme hydrolysis.

The HPLC-BA-ELISA is more time-consuming than the BA-ELISA, taking 2 days to complete, but is limited in the amount of DNA which can be analyzed only by the amount of hydrolyzed DNA which can be loaded on the HPLC column. In HPLC-fluorimetry it is critical to achieve good separation of the modified nucleobases from other components that could also fluoresce at the particular excitation and emission wavelengths used. Good separation of the nucleoside mixture is not as critical in HPLC-BA-ELISA because of the high specificity of the antibody for the carcinogen modified nucleoside.

As shown in Figure 3, reduction of NNK to NNAl is a major metabolic pathway which was observed *in vivo*, in organ cultures and during incubation of NNK with rat liver microsomal fraction (3). Interestingly, this reduction was also observed in mouse tissue early during fetal life (18). In human tissues excised from the oral cavity and upper respiratory tract and cultured *in vitro*, reduction of NNK to NNAl was also the major metabolic pathway. α-Carbon hydroxylation of NNK by cultured

human tissues was also observed, but was not as extensive as in cultured animal tissue (19).

The reoxidation of NNAl to NNK was observed with explants of A/J mouse lung (a target tissue of NNK and NNAl), although the equilibrium favored NNAl (20). The ability of NNK and NNAl to generate methylating species was compared in cultured rat nasal mucosa (Table 1). Levels of O⁶-MeGua and 7-MeGua were higher with NNK than with NNAl, suggesting that NNK is a better substrate for the activating enzymes. This hypothesis was supported by a study of the interconversion of NNK and NNAl by these explants. NNK was efficiently reduced to NNAl but the reverse reaction was not favored. Furthermore, the low level of oxidation of NNAl to NNK paralled a low level of DNA methylation observed with NNAl after 24 hr of culture. These results suggest that NNAl is not associated with the activation of NNK to DNA methylating species.

NNK, along with NNN and N'-nitrosoanatabine, are formed by N'-nitrosation of either nicotine or anatabine during curing and/or smoking of tobacco (21,22). All three N'-nitrosamines induce tumors in F344 rats, but NNK is the most potent. Even at a dose of 1 mmole/kg body weight NNK injected SC to male F344 rats induces a high percentage of nasal tumors (74%) and lung tumors (85%) but a low percentage of liver tumors (11%). In contrast, NNN induces a lower percentage of nasal tumors (56%), a low percentage of lung tumors (14%), and no liver tumors (23).

After treatment of F344 rats with NNK, methylation of the DNA at the O⁶-Gua site was observed only in organs that develop tumors (Table 3). In NNN-treated rats no O⁶-MeGua was observed in nasal mucosa or liver DNA. These results suggest that the relatively higher carcinogenic potency of NNK compared to NNN could be due to the formation of the promutagenic lesion O⁶-MeGua by NNK. However, the formation of other adducts could also mediate the carcinogenicity of NNK. A recent mutagenicity study of compounds analogous to NNK supports this hypothesis (24).

As shown in Figure 3, methyl hydroxylation of NNK would lead to the pyridyloxobutyldiazohydroxide (Compound 5). According to a whole-body autoradiographic study of rats treated with NNK, alkylation by compound 5 would take place in the mucosa of the ethmoturbinates, lateral nasal gland, bronchial mucosa, and liver (25). Whether compound 5 (Fig. 3) leads to persistent and mispairing adducts is currently being studied with monospecific antibodies and BA-ELISA.

Methodology developed in the present study will certainly be instrumental in assessing damage to human DNA by NNK and other methylating substances present in tobacco smoke and chewing tobacco.

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